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Detection of *Anaplasma phagocytophilum* genotypes that are potentially virulent for human in wild ruminants and *Ixodes ricinus* in Central Italy

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ABSTRACT

Human granulocytic anaplasmosis (HGA) is an emerging tick-borne zoonosis worldwide. As is the case for many tick-borne diseases, the epidemiological cycle is associated to the environmental conditions, including the presence of wild vertebrate reservoir hosts, vectors, climate and vegetation. In this study a total number of 87 spleen samples of wild ruminants carcasses from Central Italy, and 77 *Ixodes ricinus* collected from the same dead animals were screened for *Anaplasma phagocytophilum* by using Real Time PCR. *A. phagocytophilum* DNA was detected in 75%, 66.7% and 54.2% of the spleen samples from red deer (*Cervus elaphus*), Apennine chamois (*Rupicapra pyrenaica ornata*) and roe deer (*Capreolus capreolus*) respectively, whereas it was detected in the 31.2% of *I. ricinus*. A total of 27 positive samples were characterized by sequencing a portion of the *groEL* gene. Two *A. phagocytophilum* lineages could clearly be delineated from the phylogenetic tree. Four sequences from red deer, 2 from *I. ricinus* and 1 from Apennine chamois clustered into lineage I together with those previously described as virulent genotypes related to HGA. The presence of *A. phagocytophilum* DNA in the Apennine chamois represents the first report for this Italian endemic subspecies.

1. Introduction

Anaplasma phagocytophilum is a tick-borne pathogen that infects humans and animals. It causes a wide range of clinical symptoms including, in some cases, lethal disease. Symptoms of human granulocytic anaplasmosis (HGA) caused by *A. phagocytophilum* are non-specific and are characterized by fever, loss of appetite, muscular pain, joint pain, and in most cases patients recover without antibiotic treatment in two to three weeks, however some cases may evolve to fatal illness. The standard therapy is based on doxycycline or tetracycline treatment (Jin et al., 2012). In such instances, if undiagnosed and therefore untreated, some cases may evolve to fatal illness. The first report of HGA was notified in the USA in 1990 and then in Europe and Asia (Jin et al., 2012). In Italy, HGA was reported in Sicily (De la Fuente et al., 2005), Sardinia (Mastrandrea et al., 2006) and Friuli Venezia Giulia near to Italy's border with Austria and Slovenia (Beltrame et al., 2006; Ruscio and Cinco, 2003).

Tick-borne fever (TBF) is the disease caused by *A. phagocytophilum* in domestic ruminants. Symptoms are non-specific in cattle and sheep—the most commonly reported being high fever, loss of appetite, and anorexia. Decreased milk production, abortion and infertility may also occur (Ogden et al., 1998). The severity of the disease is often associated to secondary infections causing tick pyemia and pasteurellosis (Daniel et al., 2015; Woldehiwet, 2010). Evidence of *A. phagocytophilum* infection has been reported in dogs, horses, rodents and also in wild ruminants including Cervidae (De la Fuente et al., 2008; Liz et al., 2002; Woldehiwet, 2006), however the pathogenicity of the agent in wild ruminants is still unknown (Milner and van Beest, 2012).

A. phagocytophilum is normally transmitted by tick bite. The main vector in Europe is *Ixodes ricinus* (Rizzoli et al., 2014; Strle, 2004), however, other *Ixodes* species have been described worldwide to act as vectors (Ogden et al., 1998; Parola et al., 2005). Hosts are well documented while there is still discussion about the reservoirs of the pathogen. Indeed, in Europe *A. phagocytophilum* has been detected in *Apodemus flavicollis*, *A. sylvaticus* and *Myodes glareolus;* however, in different studies no DNA of *A. phagocytophilum* has been detected or only at low prevalence rates. Therefore the reservoir role of these rodents remains unclear (Burri et al., 2014;

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Pascucci et al., 2015; Stuen et al., 2013). Since no transovarial transmission has been demonstrated in *I. ricinus* ticks (Bakken and Dumler, 2008; Rikihisa, 2011) and because of the uncertain role played by rodents, it is reasonably accepted that the infection is maintained in the environment by a tick-ruminant cycle (Liz et al., 2000; Veronesi et al., 2011; Woldehiwet, 2010).

One of the several molecular markers used for phylogenetic studies for *A. phagocytophilum* is the *groEL* gene. This gene has high genetic heterogeneity and two distinct clusters or lineages could be delineated in Europe by sequence analysis (Lommano et al., 2014; Polin et al., 2004; Rymaszewska, 2011,2014). HGA strains are included in the lineage I together with those causing disease in horses, cats, dogs, and wild boars (Michalik et al., 2012; Petrovec et al., 2003). Strains causing TBF in domestic ruminants and described in wild cervids have been considered to be non-pathogenic for humans. However, recent findings have revealed the presence of *A. phagocytophilum* strains potentially virulent for humans in sheep, goats and also in roe and red deer (Carpi et al., 2009; Jahfari et al., 2014; Katargina et al., 2011; Petrovec et al., 2002; Rymaszewska, 2014).

In the present study we aimed to assess the presence of *A. phagocytophilum* infection in wild ruminants and feeding ticks in Central Italy. Moreover, by sequencing a portion of the *groEL* gene, we reported the spreading in this region of strains previously associated with HGA in humans.

2. Materials and methods

2.1. Sample collection

From August 2011 to September 2014, 87 carcasses of wild ruminants were found in different areas of Abruzzo region (Italy) by forest rangers and veterinarians of National Parks. Roadkills represent more than 80% of the case fatality. Twelve red deer (*Cervus elaphus*), 9 Apennine chamois (*Rupicapra pyrenaica ornata*), 59 roe deer (*Capreolus capreolus*), 1 fallow deer (*Dama dama*) and 6 mouflon (*Ovis musimon*) were dissected and observed. Spleens collected from each animal were homogenized in PBS and immediately used for DNA extraction.

Simultaneously, feeding ticks were collected from each animal and stored in plastic vials containing ethanol 70% (v/v). A total of 77 engorged female ticks were identified to be *I. ricinus* by stereomicroscopy as previously described (Manilla, 1998).

Once identified, the ticks were transferred individually into the cartridges, provided by the DNA Maxwell 16 Tissue Purification Kit (Promega), for automated DNA extraction and purification following the manufacturer's instructions. DNA extraction from spleen homogenates was carried out on 300 μL by using the same extraction kit used for ticks.

2.2. Real Time PCR

A Real Time PCR assay targeting the *msp2* gene was used to detect *A. phagocytophilum* in tick and spleen samples (Courtney et al., 2004). The reaction mixture consisted of 20 μL containing 2X TaqMan Fast Universal PCR Master Mix (Applied Biosystems), 250 nM of TaqMan probe, 900 nM of each primer, nuclease-free water and 5 μL DNA. The reaction protocol used was: 20 s at 95 °C followed by 40 cycles of 1 s at 95 °C and 20 s at 60 °C. The test was performed on the 7900HT Fast Real Time PCR System (Applied Biosystems) and analysed by SDS 2.4 (Applied Biosystems). The Real Time PCR assays were performed including a plasmid containing a portion of *msp2* gene as positive control and No Template Control (NTC) as negative control.

2.3. groEL gene partial sequencing

Positive samples were further investigated by sequencing an hyper-variable fragment (546 bp) of the *groEL* gene. Primers were specifically designed for this study by annealing different *A. phagocytophilum groEL* sequences retrieved on GenBank. 5 μL of purified DNA were amplified in a 50 μL reaction mixture containing 300 nM of each primer (AphGroEL_fwd 5′-GCTGAAAAAATGCTGGTGGAA-3′; AphGroEL_rev 5′-AACTTCGCTGGATCCACCAA-3′), 200 μM dNTPs (Promega), 2.5 mM $MgCl_2$ Solution (Applied Biosystem), 0.125 U/μL AmpliTaq Gold™ (Applied Biosystem) and 1X PCR Buffer II (Applied Biosystem). Amplifications were carried out in a GeneAmp PCR System 9700 (Applied Biosystems) under the following conditions: initial denaturation 10′ at 94 °C, followed by 45 cycles of denaturation (30″ at 94 °C), annealing (30″ at 56 °C), and extension (30″ at 72 °C). The final extension was completed at 72 °C for 7′. PCR products were purified using the GeneAll Expin™ PCR Kit (GeneAll) and sequenced by the BigDye Terminator v.3.1 (Applied Biosystems). Analyses were performed on the 3130 XL Genetic Analyzer (Applied Biosystems). Raw sequence data were assembled using Contig Express (Vector NTI suite 9.1; Invitrogen).

The *groEL* sequences coming straight from DNA samples of the present study and several sequences of *A. phagocytophilum* isolated worldwide available in GenBank were used for phylogenetic analysis. Sequence alignment and phylogenetic analysis were conducted using MEGA version 6 and the evolutionary distances were computed using the maximum likelihood method. Statistical support was provided by bootstrapping over 1000 replicates and bootstrap values are indicated at the corresponding node (Tamura et al., 2013).

3. Results

A total of 47 out of 87 (54%; IC 95% 43.6–64.1%) spleen samples resulted positive for *A. phagocytophilum* DNA by Real Time PCR.

Twenty-four out of 77 (31.2%; IC 95% 21.9–42.2%) ticks turned out to be positive for pathogen DNA. Ten (16%; IC 95% 9.5–28.5%) roe deer and 4 (33%; IC 95% 13.9–61.4%) red deer were infested with *I. ricinus*. Results of both spleen samples and ticks are summarized in Table 1. Moreover, a total of 27 DNA positive samples were amplified by the specific PCR for the *groEL* gene and then sequenced while the other positive samples, showing a Ct value >32 in Real Time PCR, failed in the *groEL* gene amplification. The sequences were submitted to GenBank (Accession Number from KR019967 to KR019993). A phylogenetic tree was constructed by using the 27 obtained sequences together with 39 other *groEL* partial gene sequences retrieved from GenBank. The percentage similarity within *A. phagocytophilum* sequences obtained in this study varies from 95.1% to 100%. A total of seven sequences from 4 red deer, 2 *I. ricinus* and 1 Appennine chamois clustered into lineage I together with those coming straight from human cases. The other 20 *A. phagocytophilum* sequences from 13 roe deer, 1 red deer and 6 *I. ricinus* grouped into the lineage II were not related to HGA. GenBank accession numbers, host species and related lineages are reported in Fig. 1. Simultaneous infection of *A. phagocytophilum* in both ticks and animal hosts was reported in 1 red deer/tick and 4 roe deer/ticks association. However, only the *I. ricinus* feeding on *Cervus elaphus* have shown the identical partial sequence of *A. phagocytophilum groEL* (respectively KR019980 and KR019986). In contrast, all the ticks collected on *Capreolus capreolus* have shown different *A. phagocytophilum groEL* partial sequence compared to that one detected in the host.

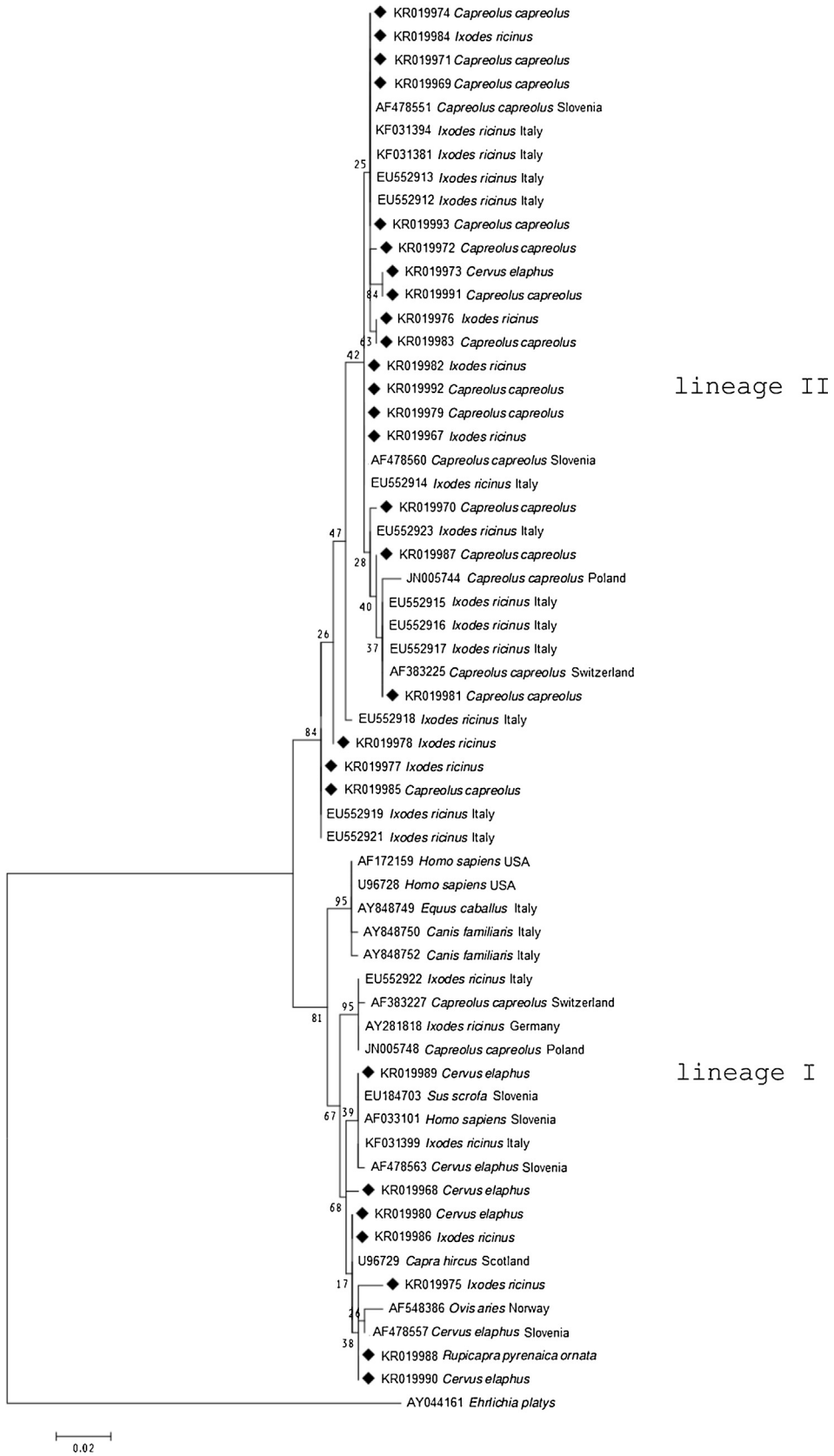

Fig. 1. Phylogenetic tree based on *groEL* partial gene sequences (449 bp) of *A. phagocytophilum* found in wild ruminants and ticks feeding on the same animals, in comparison to sequences obtained from GenBank database. ♦ New Italian sequences obtained in this study.
The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 66 nucleotide sequences. There were a total of 449 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

Table 1
Anaplasma phagocytophilum DNA amplification by Real Time PCR in wild ruminant spleens and *Ixodes ricinus* ticks.

Species	Spleen			*Ixodes ricinus*		
	tested	positive (%)	IC 95%	tested	positive (%)	IC 95%
red deer	12	9 (75%)	46.2–90.9%	18	2 (11.1%)	3.4–33.1%
Apennine chamois	9	6 (66.7%)	34.7–87.8%	0	0	
roe deer	59	32 (54.2%)	41.6–66.3%	59	22 (37.3%)	26–50.1%
fallow deer	1	0		0	0	
mouflon	6	0		0	0	
Total	87	47 (54%)	43.6–64.1%	77	24 (31.2%)	21.9–42.2%

4. Discussion and conclusions

This study investigated the circulation of *A. phagocytophilum* in ticks and wild ruminants collected in the Abruzzo region, which is home to several national and regional parks and protected areas. Spleen samples were collected from roe deer, red deer, Apennine chamois, mouflon and fallow deer. Ticks collected from the carcasses were screened and tested positive for *A. phagocytophilum*. The investigation revealed the widespread circulation of the agent in enzootic cycles in Central Italy where the main vector *I. ricinus* is present.

Roe deer is known to be an important reservoir host for *A. phagocytophilum* (Alberdi et al., 2000; De la Fuente et al., 2008; Liz et al., 2002). In this study, the infection rate of 54.2% observed in roe deer is in accordance to other studies conducted in different European countries (Polin et al., 2004; Víchová et al., 2014). Higher prevalence rates 86% in Poland and 98.9% in Germany were reported by Petrovec et al. (2002) and Overzier et al. (2013), respectively. Conversely, a survey carried out in a game reserve in the Andalusia region (Spain) highlights a lower prevalence (18%) as well as in the UK and northern Italy in which the prevalence reported was lower than 30% in both studies (Alberdi et al., 2000; Beninati et al., 2006; De la Fuente et al., 2008).

Authors link the spread of *I. ricinus* to the recent increase of the roe deer population in Central Italy as observed in the northeast of the country (Chemini et al., 1997). Roe deer usually seek for food in grazing areas that are humid and protected from the sunlight. These eco-climatic conditions are also favourable for *I. ricinus* (Manilla, 1998; Maroli et al., 1995). It is likely that the strict association between roe deer and *I. ricinus* could affect the spread of the infection in the study area, as described for other European countries (Alberdi et al., 2000; De la Fuente et al., 2008). However, due to the sampling size and the tick collection on dead animals, the provided information are not exhaustive and further investigations are needed to make available more significant evidences.

This study also considered other wild ruminants. High infection rate was also observed in red deer (75%), even though the number of samples is too limited to provide a representative value of prevalence in the population. However, high prevalence in red deer has been reported in the Northeast of Poland (50.9%) and Slovenia (86%) (Hapunik et al., 2011; Petrovec et al., 2002).

High rate of infection was similarly observed in Apennine chamois (66.7%) for which no data about the infection of *A. phagocytophilum* is available in literature. Indeed, *A. phagocytophilum* has been reported only in Alpine chamois (*Rupicapra rupicapra*) (Liz et al., 2002). Apennine chamois is an endangered subspecies endemic of Central Italy, thus the possible impact of anaplasmosis on the survival of these subspecies should be considered when drawing conservation strategies. In fact, although infection in wildlife often assumes subclinical features, it can nevertheless influence population dynamics if reproductive success is affected (Milner and van Beest, 2012).

However, since only few samples were analysed, the infection rate observed in the Apennine chamois could be overestimated. Therefore, further investigation to consider the possible epidemiological role played by this wild ruminant is recommended. No *I. ricinus* was found in any of the Apennine chamois investigated. This tick is generally associated to deciduous mesophilous woodlands while the Apennine chamois usually lives in rocky high pastures. However, different authors have reported Alpine and Pyrenean chamois infested with *I. ricinus* (Davoust et al., 2012; Liz et al., 2002). A more extended sampling of feeding ticks on Apennine chamois population during the appropriate season could fill the gap of data for this topic.

Nonetheless, the main issue of the present work was to genotype *A. phagocytophilum* strains in reservoir hosts and vectors in order to asseverate the infectiousness and the potential risk for human health. Indeed, genotyping of bacterial strains not only provides detailed information on intraspecific diversity that is useful for epidemiological purposes but also enables prevention for disease in humans. Different approaches have been used to genotype agents of emergent zoonosis aimed to explain pathogenicity, antibiotic resistance and virulence (Aminov and Mackie, 2007; Di Domenico et al., 2014; Field et al., 2004). In the present study, genetic variants of *A. phagocytophilum* were characterized by partial *groEL* sequencing. Other targets have also been used to genotype this intracellular bacterium, however the *groEL* seems to be particularly suitable for the purpose because of its high variability (Carpi et al., 2009; Jahfari et al., 2014; Rymaszewska, 2008, 2014). Two *A. phagocytophilum* clusters, or lineages, could clearly be delineated from the phylogenetic tree accordingly to previous studies (Lommano et al., 2014; Polin et al., 2004; Rymaszewska, 2011, 2014). Two additional lineages were described as well (Jahfari et al., 2014), which were most likely associated to birds and rodents, and thus not considered in this study. Recently, authors reported the circulation of human pathogenic strain in questing *I. ricinus* ticks in Italy. However, they did not find this strain in any of the host-fed ticks analysed (Baráková et al., 2014). In a PCR positive red deer spleen sample, the *groEL* gene partial sequence (KR019989) was analysed resulting identical to the sequence from the tick collected in Italy (KF031399) and from human HGA in Slovenia (AF033101) (Baráková et al., 2014; Petrovec et al., 1999), that is the only sequence of *A. phagocytophilum* identified in humans in Europe. However, two more sequences have been published from USA patients clustering in the same lineage I. All sequences grouped in this clade show a conserved substitution in the nucleotide sequence at position 242 causing an amino-acid substitution, the so called Serine variants Vgro-S (Rymaszewska, 2014). Since any *A. phagocytophilum* strain that shows this substitution in the GroEL sequence is potentially dangerous for humans, more extensive studies have to be performed to define the possible role of red deer and Apennine chamois in the maintenance of human pathogenic strains in the environment.

On the other hand, *A. phagocytophilum* sequences from all 13 roe deer clustered in the lineage II accordingly to the role as wildlife

reservoir for their own specific *A. phagocytophilum* ecotypes that were not involved in human cases (De la Fuente et al., 2008; Petrovec et al., 2002).

This study represents the first report of *A. phagocytophilum* in Central Italy. Additional investigations on pathogenic pathways of strains involved in HGA cases may help scientists to assess risk factors for human health.

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